TITLE OF THE INVENTION

AUTOANTIGENIC FRAGMENTS, METHODS AND ASSAYS

CROSS-REFERENCE TO RELATED APPLICATIONS

Not applicable.

STATEMENT REGARDING FEDERALLY-SPONSORED R&D

This invention was made in part under Federally Sponsored Research. The U.S. Government may have certain rights in this invention.

REFERENCE TO MICROFICHE APPENDIX

Not applicable.

FIELD OF THE INVENTION

This invention relates to the production of autoantigenic fragments from autoantigens and the uses of the autoantigenic fragments.

BACKGROUND OF THE INVENTION

The mature immune system of animals differentiates between self-molecules and non-self-molecules and mounts an immune response only against the latter. The immune system learns which molecules are self through constant exposure to those molecules that are normally a part of the animal. Thus, the mature immune system is tolerized to the presence of molecules that are self. However, the immune system is not tolerized to molecules that are newly presented in the animal. These molecules can be antigens and thereby stimulate an immune response against them. Commonly, newly presented antigens are from an extracorporeal source, such as an infection. In this case, the immune response helps to destroy the source of the antigens and thereby clear the infection from the body.

Newly presented antigens are produced *in vivo* through the degradation of cellular components. When the immune system recognizes these degradation products of self molecules as "non-self"

antigens, an immune response can be mounted against them and an autoimmune disease can develop. Thus, these antigens are members of the class of molecules generally referred to as autoantigens and the antibodies produced against them are referred to as autoantibodies. For clarity herein, autoantigen is used to refer to the complete self molecule as found in the body. Autoantigenic fragment is used to refer to the degradation product of the autoantigen. Thus it is when an epitope is presented to the immune system as autoantigenic fragment that an immune response is elicited. Once elicited, the immune response can target the autoantigenic fragment, the autoantigen, or both.

Autoimmune diseases are diseases in which a specific immune response to self-molecules occurs, often leading to tissue and organ damage and dysfunction. The diseases can be organ-specific (e.g. Type I diabetes mellitus, thyroiditis, myasthenia gravis, primary biliary cirrhosis) or systemic in nature (e.g. systemic lupus erythematosus, rheumatoid arthritis, polymyositis, dermatomyositis, Sjogrenís syndrome, scleroderma, and graft-vs.-host disease).

One source of autoantigenic fragments is cleavage of an autoantigen during apoptosis. Apoptosis is a morphologically and biochemically distinct form of cell death that occurs in many different cell types during a wide range of physiologic and pathologic circumstances (reviewed in (Jacobson *et al.*, 1997; Thompson, 1995; White, 1996)). Studies report that specific proteolysis catalyzed by a novel family of cysteine proteases is of critical importance in mediating apoptosis (Chinnaiyan and Dixit, 1996a; Martin and Green, 1995; Thornberry and Molineaux, 1995). These proteases (termed caspases), cleave downstream substrates after a consensus tetrapeptide sequence ending with aspartic acid (P_1). The caspases are synthesized as inactive precursors that require specific proteolytic cleavage after an aspartic acid residue for activation (reviewed in (Nicholson and Thornberry, 1997)).

Granzyme B, a serine protease found in the cytoplasmic granules of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells, has a similar requirement to caspases, for aspartic acid in the substrate P_1 position (Odake *et al.*, 1991; Poe *et al.*, 1991). Studies have reported

that granzyme B plays an important role in inducing apoptotic nuclear changes in target cells during granule exocytosis induced cytotoxicity (Darmon *et al.*, 1996; Heusel *et al.*, 1994; Sarin *et al.*, 1997; Shresta *et al.*, 1995; Talanian *et al.*, 1997).

Granzyme B is described as catalyzing the cleavage and activation of several caspases (Chinnaiyan *et al.*, 1996b; Darmon *et al.*, 1995; Duan *et al.*, 1996; Fernandes-Alnemri *et al.*, 1996; Gu *et al.*, 1996; Martin *et al.*, 1996; Muzio *et al.*, 1996; Quan *et al.*, 1996; Sarin *et al.*, 1997; Song *et al.*, 1996a; Srinivasula *et al.*, 1996; Talanian *et al.*, 1997; Wang *et al.*, 1996). Granzyme B also initiates caspase-independent pathways which contribute to target cell death. However, while several candidates for these additional pathways exist, they remain largely undefined (Sarin *et al.*, 1997; Talanian *et al.*, 1997).

One candidate pathway is the direct proteolysis of death substrates by granzyme B, although efficient non-caspase cellular substrates for this protease have not yet been identified. Initial studies have indicated that the cleavage of PARP, U1-70kDa and lamin B observed during granzyme B-induced cell death is catalyzed by caspases, rather than directly by granzyme B (Darmon *et al.*, 1995; Martin *et al.*, 1996; Talanian *et al.*, 1997), but the effects of granzyme B on other caspase substrates *in vitro* and during granule-induced cytotoxicity have not been extensively studied.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Granzyme B cleaves purified DNA-PK_{CS}, NuMA, PARP and caspases 3 and 7 *in vitro* with different efficiencies. Reactions containing 0-12.5nM granzyme B (for DNA-PK_{CS} and NuMA)or 0-50 nM granzyme B (for caspases 3 and 7 and PARP) were performed as described in Example 1. Cleavage fragments were detected by fluorography or immunoblotting (monoclonal antibody 18-2 was used to detect DNA-PK_{CS}). On the right side of each panel, the SDS-PAGE migration positions of the intact molecules are denoted by arrows, and the fragment sizes are indicated.

FIG. 2. Cleavage of autoantigens *in vitro* with purified granzyme B or caspase-3 yields different fragments. Reactions

containing 30nM purified DNA-PK_{CS} (lanes 1-3), endogenous DNA-PK_{CS} and NuMA in HeLa lysate (lanes 4-9) or [^{35}S]methionine-labeled PARP (lanes 10-12) were incubated with the following amounts of granzyme B: 1.25nM (lane 2); 12.5nM (lanes 5 & 8) or 50nM (lane 11). Similar experiments were performed with these substrates using the following amounts of caspase-3: 42pM (lanes 3 &12) or 100pM (lanes 6 & 9). Reaction mixtures were incubated at 37°C for 15 min. (granzyme B reactions) or 60 min. (caspase-3 reactions). Note that 100nM Ac-DEVD-CHO was added when granzyme B was used to cleave DNA-PK_{CS} and NuMA in HeLa cell lysates, to prevent activation of HeLa cell caspases. Intact and cleaved NuMA and DNA-PK_{CS} were visualized by immunoblotting, and PARP was visualized by autoradiography. In the case of DNA-PK_{CS}, blots obtained using monoclonal antibodies 25-4 (directed against the C-terminus) and 18-2 (directed against the N-terminus) are shown in lanes (1-3) and (4-6), respectively.

FIG. 3. Kinase activity of DNA-PK_{CS} is abolished after cleavage with granzyme B. Kinase assays were performed using intact DNA-PK_{CS} (lanes 1 & 2) or granzyme B-cleaved DNA-PK_{CS} (lanes 3 & 4) in the absence (lanes 2 & 4) or presence (lanes 1 & 3) of 10μg/ml of DNA. DNA-PK_{CS} itself was omitted from the otherwise complete kinase reaction mix as a control in lane 5. Phosphorylation of SP1 substrate was detected by autoradiography.

FIG. 4. Endogenous DNA-PK_{CS} and NuMA in HeLa cell lysates are cleaved in a caspase-independent manner after adding purified granzyme B and incubating *in vitro*. 12.5 nM purified granzyme B (lanes 5-7) or 105 pM purified caspase-3 (lanes 2-4) were added to lysates of control HeLa cells, in the presence of 100 nM Ac-DEVD-CHO (lanes 3 & 6) or 100nM Ac-YVAD-CHO (lanes 4 & 7). After incubating at 37°C for 60 min, the reactions were terminated. DNA-PK_{CS}, NuMA and PARP were detected by immunoblotting as described in Example 1 (monoclonal antibody 18-2 was used to detect DNA-PK_{CS}). Equal amounts of protein were electrophoresed in each lane.

FIG. 5. Granzyme B cleaves DNA-PK_{CS} at VGPD^{2698}-F^{2699} and DEVD^{2712}-N^{2713}. A [^{35}S]methionine-labeled wild-type (wt) DNA-

PK_{cs} polypeptide (DNA-$PK_{cs}^{2566-2928}$) and two radiolabeled polypeptides containing mutations in the P_1 positions of the predicted granzyme B cleavage site ($D^{2698}A$) and the known caspase-3 cleavage site ($D^{2712}A$), were generated as described in Example 1. These polypeptides were incubated in the absence of added proteases (lanes 1, 4 & 7), or in the presence of 8nM recombinant caspase-3 (lanes 2, 5 & 8) or 8 nM purified granzyme B (lanes 3, 6 & 9) for 60 min at 37°C. After terminating the reactions, samples were electrophoresed, and the intact polypeptide and the cleavage products were detected by fluorography. Fragment sizes of 18kDa (a), 28kDa (b), 20kDa (c) and 26kDa (d) were generated (see lower panel for schematic representation).

FIG. 6. Endogenous DNA-PK_{cs}, NuMA and PARP are cleaved after *in vivo* incubation of intact K562 cells with YT cell granule contents. K562 cells were incubated for 30 min at 37°C in the absence (lanes 1-3) or presence (lane 4) of 100 μM Ac-DEVD-CHO. Aliquots of these cell suspensions (each containing 3 x 10^5 K562 cells) were then further incubated for 90 min at 37°C in the presence of 1mM Ca^{2+} (lane 1), 1 mM EDTA + YT cell granule contents (lane 2) or 1 mM Ca^{2+} + YT cell granule contents (lanes 3 & 4). After terminating the reactions, DNA-PK_{cs}, NuMA and PARP were detected by immunoblotting as described in the Example 1 (Patient serum G.A. was used to blot DNA-PK_{cs}).

FIG. 7. Granzyme B-specific fragment of DNA-PK_{cs} is generated in K562 cells attacked by LAK cells. Fas-negative K562 target cells were preincubated in the absence (lanes 1-3) or presence (lane 4) of 100μM Ac-DEVD-CHO for 1 hr, followed by co-incubation for a further 4 hr at 37°C (effector:target ratio 5:1). After terminating the reactions, the following numbers of cells were electrophoresed in each gel lane: 1.7 x 10^6 LAK cells (lane 1); 0.34 x 10^6 K562 cells (lane 2); 1.7 x 10^6 LAK cells plus 0.34 x 10^6 K562 cells (lanes 3 & 4). DNA-PK_{cs} and PARP were detected by immunoblotting; patient serum G.A. was used to detect DNA-PK_{cs}.

FIG. 8. Ac-DEVD-CHO-insensitive nuclear morphologic changes are induced in intact HeLa cells after *in vivo* incubation with

YT cell granule contents. HeLa cells were incubated with YT cell granule contents for 1hr at 37°C in the absence (8A & 8B) or presence (8C) of 100μM Ac-DEVD-CHO as described in Experimental Procedures. After fixation and permeabilization, cells were stained with antibodies to PARP, as well as propidium iodide (PI) and DAPI. Antibody staining was visualized with FITC-goat anti-human antibodies. Merged images of antibody staining (green), PI staining (red) and DAPI staining (blue) are presented. (8A, 8B): YT cell granule contents induce prominent PI-rich surface blebs, nuclear condensation and fragmentation, with a characteristic redistribution of PARP to the rim of the condensing nucleus (8A) or apoptotic bodies (8B, arrows). (8C): Granule contents induce marked nuclear condensation even in the presence of Ac-DEVD-CHO (arrows); an adjacent, normal HeLa cell nucleus that has not yet undergone morphologic change is shown for comparison (arrowhead). Note that treatment with Ac-DEVD-CHO abolishes the formation of PI-rich surface blebs, nuclear fragmentation, and the redistribution of PARP. Size bar: 8A: 4.4 μm; 8B: 4.0 μm; 8C: 6.6 μm.

FIGS. 9A-9B shows a 2101 amino acid sequence of NuMA as found on Entrez at ACCESSION 284337; PID g284337; DBSOURCE PIR: locus A42184.

FIGS. 10A-10B shows a 2115 amino acid sequence of NuMA as found on Entrez at ACCESSION 107227, PID g107227; DBSOURCE PIR: locus S23647.

FIGS. 11A-11C shows the amino acid sequence of DNA PK_{CS} as found on Entrez at ACCESSION 1362789; PID g1362789; DBSOURCE PIR: locus A57099.

FIGS. 12A-12B shows the amino acid sequence of PARP as found on Entrez at ACCESSION 130781; PID g130781; DBSOURCE SWISS-PROT: locus PPOL_HUMAN, accession P09874 (listing only references 1 & 2 of 12).

SUMMARY OF THE INVENTION

The present invention provides autoantigenic fragments and methods for their use in the treatment of autoimmune disease. Also

provided are assays for detecting an autoimmune condition in an animal, including the presence of an autoimmune disease.

Caspase-mediated proteolysis of downstream substrates is a critical element of the central execution pathway common to all forms of apoptosis studied to date. While cytolytic lymphocyte granule-induced cell death activates this caspase-dependent pathway, recent studies have also provided evidence for caspase-independent pathways in this form of cell death. However, non-caspase substrates for granzyme B (and potentially other granule proteases) during granule-induced cell death have not previously been defined. The present invention makes use of the observation that cellular components are directly and efficiently cleaved by granule contents, including in particular granzyme B, *in vitro* and *in vivo*, and that this cleavage leads to the generation of unique autoantigenic fragments not observed during other forms of apoptosis. This direct, caspase-independent ability of granzyme B to cleave downstream death substrates to autoantigenic fragments is an apoptotic effector mechanism which is insensitive to inhibitors of the signaling or execution components of the endogenous apoptotic cascade.

An aspect of this invention is a composition that includes at least one autoantigenic fragment. The autoantigenic fragment is produced by the action of a granule enzyme on an autoantigen. In a preferred embodiment, the enzyme is a granule enzyme of CTL, NK or LAK cell granules. In a most preferred embodiment, the enzyme is granzyme B and the antigenic fragment is produced by the cleavage of the autoantigen by granzyme B at a site that is not cleaved by a caspase. In a preferred embodiment the autoantigen is DNA PK_{CS}, PARP or NuMA. In a most preferred embodiment, the autoantigenic fragment is one or more of DNA-PK_{CS} from amino acid 2699 to 4096; DNA-PK_{CS} from amino acid 3211to 4096; PARP from amino acid 1 to 537; PARP from amino acid 538 to 1004; NuMA from amino acid 412 to 2111 and NuMA from amino acid 1 to 1799.

An aspect of this invention is a pharmaceutical composition made with one or more purified and isolated autoantigenic fragments. In a preferred embodiment, the autoantigenic fragment has at least one end derived from granzyme B cleavage at a site in the autoantigen that is

not cleaved by a caspase. A pharmaceutically acceptable carrier is also included. In a preferred embodiment the composition includes one of more of the following autoantigenic fragments: DNA-PK$_{CS}$ from amino acids 2699 to 4096; DNA-PK$_{CS}$ from amino acids 3211to 4096; PARP from amino acid 1 to 537; PARP from amino acids 538 to 1004; NuMA from amino acids 412 to 2111 and NuMA from amino acids 1 to 1799. In another preferred embodiment the pharmaceutical composition includes one or more autoantigenic fragments derived from a malignant cell.

An aspect of this invention is a method of treating a patient in need of treatment for an autoimmune disease by administering to the patient an autoantigenic fragment of this invention. The autoimmune disease can be organ specific, *e.g.*, Type I diabetes mellitus, thyroiditis, myasthenia gravis, primary biliary cirrhosis, or systemic in nature *e.g.* systemic lupus erythematosus, rheumatoid arthritis, polymyositis, dermatomyositis, Sjogrenís syndrome, scleroderma, and graft-vs.-host disease. In one preferred embodiment, the treatment is therapeutic. For example, a patient suffering from an immune disease can be administered an autoantigenic fragment by contacting the sera of the patient with the fragment under conditions that allow the binding of autoantibodies in the sera to bind to the fragment. In this embodiment, the level of autoantibodies circulating in the patient can be reduced. In another embodiment the treatment is prophylactic. In this embodiment, a patient who is at risk of developing an autoimmune disease is tolerized to at least one autoantigenic fragment. Thereafter, the risk of, or severity of an autoimmune disease arising upon the later production of the autoantigenic fragment *in vivo*, is reduced or eliminated. In a preferred embodiment, a patient is tolerized by identifying a target tissue to which an autoimmune disease can arise, providing at least one granule enzyme, contacting the granule enzyme with cells from the target tissue to produce autoantigenic fragments of autoantigens present in the cells. The autoantigenic fragments are then administered to the patient to tolerize the patient to the presence of the fragments. In preferred embodiments, the autoantigens can be partly or wholly purified from the cells of the target tissue. The granule enzyme can also

be partly or wholly purified before contacting with the autoantigens. The enzyme can also be made by recombinant methods. In a preferred embodiment, the autoantigenic fragments are partly or wholly purified before they are administered to the patient. In prophylactic methods of tolerizing, the autoantigenic fragments are administered in pharmaceutically acceptable compositions that are designed not to raise an immune response to the fragments, *i.e.*, no immunostimmulatory adjuvants are administered with the fragments. In a preferred embodiment, the treatment uses one or more of the following autoantigenic fragments: DNA-PK_{CS} from amino acids 2699 to 4096; DNA-PK_{CS} from amino acids 3211to 4096; PARP from amino acid 1 to 537; PARP from amino acids 538 to 1004; NuMA from amino acids 412 to 2111 and NuMA from amino acids 1 to 1799.

An aspect of this invention is a method of treating a patient in need of treatment for a malignancy. In a preferred embodiment, at least one enzyme of a lymphocyte granule is contacted with the malignant cells from the patient. This can produce a mixture containing autoantigenic fragments derived from the malignant cells. The fragments are administered to the patient, preferably with an adjuvant, to stimulate an immune response against the malignant cells.

An aspect of this invention is an assay for the detection of an autoantigenic fragment in a patient. In one embodiment, the presence or absence of the fragment in a patient sample is an indication of the presence or absence of an autoimmune condition in the patient. In a preferred embodiment, a sample from the patient is contacted with an antibody that specifically binds to a cryptic epitope of an autoantigenic fragment. Preferably, the fragment has at least one terminus derived from the cleavage of an autoantigen by granzyme B at a site that is not cleaved by a caspase. The presence or absence of the binding of the antibody to the autoantigenic fragment is then assessed as an indication of the presence or absence of an autoimmune condition in a patient. In an alternative embodiment, the detection of an antibody that binds an autoantigenic fragment is an indication of the presence or absence of an autoimmune condition in the patient. In this embodiment a sample from the patient is contacted with an autoantigenic fragment having at

least one terminus derived from cleavage by a granule enzyme. Detection of the presence or absence of the binding of an antibody in the sample to the autoantigenic fragment is an indication of the presence or absence of an autoimmune condition in the patient.

An aspect of this invention is a method of making an autoantigenic fragment from an autoantigen. In a preferred embodiment, one isolates cells containing at least one autoantigen and contacts the cells with a lymphocyte granule enzyme to produce a mixture containing at least one autoantigenic fragment. In a further embodiment one isolates at least one autoantigenic fragment from the mixture. In a preferred embodiment one purifies at least one autoantigen and contacts the purified autoantigen with granzyme B. In a further preferred embodiment, one purifies one or more of the following autoantigens for contacting with granzyme B: DNA-PK_{CS}, PARP and NuMA. In each embodiment the granule enzyme can isolated from the granules of a lymphocyte, *e.g.*, a cytotoxic T lymphocyte (CTL), a natural killer cell (NK), a lymphokine activated killer cell (LAK) or cells of the YT cell line.

In all aspects of this invention, granzyme B can be used in particular embodiments. the enzyme can be purified from the granules of granule containing lymphocytes or can be prepared by recombinant techniques.

Definitions:

As used herein, "treatment" includes the therapeutic or prophylactic application of a composition to a patient. A treatment can prevent, moderate or cure a disease in the patient. A disease is moderated in a patient when the treatment lessens the severity or frequency of at least one symptom associated with the disease. A treatment can moderate a disease by: (1) prophylactic administration of a composition to a patient free of a disease to lessen the impact of at least one symptom of the disease when it does occur or (2) therapeutic administration to a patient having a disease to lessen at least one symptom of the disease.

As used herein, a “patient” is an animal, particularly including a human.

As used herein, an “autoimmune condition” is the presence of, or the predisposition for the development of, an autoimmune disease or an autoimmune response in a patient.

As used herein, an “autoantigen” is a cellular molecule and usually is a protein. An autoantigen is typically not antigenic because the immune system is tolerized to its presence in the body under normal conditions. An autoantigen will typically include at least one cryptic epitope. An autoantigen can be produced by natural cells, using recombinant methods, or through chemical synthesis, as appropriate.

As used herein, an “autoantigenic fragment” is a degradation product of an autoantigen. An autoantigenic fragment is antigenic because the immune system is not tolerized to its presence in the body. Autoantigenic fragments usually display cryptic epitopes to the immune system. An autoantigenic fragment can be produced by natural cells, through the action of at least one granule enzyme in a cellular or cell free system, using recombinant methods, or through chemical synthesis, as appropriate.

As used herein, an “autoantibody” is an antibody produced by the immune system of an animal in response to the present of an autoantigenic fragment. An autoantibody can bind to the autoantigenic fragment, the autoantigen from which the fragment is derived, or both.

As used herein, a “granule containing lymphocyte” is meant to include all lymphocytes that contain granules. In particular, the term is used to include the family of cell types sometimes referred to as cytotoxic lymphocytes, to include cell lines derived from these cells and to include cytotoxic lymphocyte-like cell lines, preferably the YT cell line. Preferred cells are the granule containing lymphocytes known in the art as cytotoxic T lymphocytes (CTL), natural killer cells (NK) and lymphokine activated killer cells (LAK).

As used herein, a “lymphocyte granule enzyme” or “granule enzyme” is an enzyme that is found in the granules of a granule containing lymphocyte. A granule enzyme can be purified from a lymphocyte granule by methods commonly employed in the art of

protein purification. Additionally, a granule enzyme can be prepared by cloning the gene for the enzyme and the enzyme then is prepared using methods commonly used in the production of recombinant enzymes.

As used herein, "purified" and/or "isolated" are used interchangeably to stand for the proposition that the protein(s) and polypeptide(s), or respective fragment(s) thereof in question has been removed from its *in vivo* environment. A protein or fragment thereof is considered "purified" and/or "isolated" when it is obtained at a concentration at least about five-fold to ten-fold higher than that found in nature. A protein or fragment thereof is considered substantially pure if it is obtained at a concentration of at least about 100-fold higher than that found in nature. A protein or fragment thereof is considered essentially pure if it is obtained at a concentration of at least about 1000-fold higher than that found in nature. A protein is sometimes referred to as partly purified if it is at least purified or isolated but it is not essentially pure. A chemically synthesized protein is considered to be substantially purified when purified from its chemical precursors. A purified or isolated protein can be manipulated by the skilled artisan, such as but not limited to obtaining the protein or protein fragment in quantities that afford the opportunity to generate polyclonal antibodies, monoclonal antibodies, amino acid sequencing, and peptide digestion. Therefore, the autoantigenic fragments claimed herein can be present in cell lysates or in a substantially or essentially pure form.

Abbreviations:

Ac-DEVD-CHO, N-(N-Ac-Asp-Glu-Val)-3-amino-4-oxobutanoic acid; Ac-YVAD-CHO, N-(N-Ac-Tyr-Val-Ala)-3-amino-4-oxobutanoic acid; CTL, cytotoxic T lymphocytes; DNA-PK_{CS}, DNA-dependent protein kinase catalytic subunit; LAK cells, lymphokine-activated killer cells; NK cells, natural killer cells; NuMA, nuclear mitotic apparatus protein; PARP, poly(ADP-ribose)polymerase; PI, propidium iodide -

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides autoantigenic fragments and methods for their use in the treatment of autoimmune disease. Also provided are assays for detecting an autoimmune condition in an animal, including the presence of an autoimmune disease.

The present invention makes use of the discovery that a class of previously unrecognized autoantigenic fragments is generated during the form of apoptosis triggered by the action of the contents of lymphocyte granules on cells. In particular, enzymes found in lymphocyte granules are discovered to cleave proteinaceous cellular autoantigens to yield previously unrecognized autoantigenic fragments. Granzyme B is found to be an important granule enzyme in this generation of autoantigenic fragments. This enzyme was previously known to cleave some of the pro-caspase enzymes to yield active caspases. Granzyme B is now found to directly cleave certain of the substrates of the caspases at different sites to produce novel autoantigenic fragments.

The present invention provides a method of producing granzyme generated autoantigenic fragments *in vitro* in several ways. In a preferred embodiment, purified granzyme B can be contacted with purified substrates to produce the autoantigenic fragments. Partly or wholly purified enzyme can also be used on partially or wholly purified substrates or cellular lysates containing the substrates. In this case, the autoantigenic fragments can be purified after cleavage of the substrates. Additionally, the contents of granules can be used to produce autoantigenic fragments of cellular components, including the autoantigenic fragments of caspase substrates created by the action of granzyme B, by application of granule contents, or purified substrates or tissue samples isolated from a patient. In some embodiments, the cells of the tissue can be disrupted by lysis or mechanical breakage to release the contents of the cells before contacting the cells with the contents of the granules.

The autoantigenic fragments provided herein can be used in a treatment to tolerize a patient to the presence of the autoantigenic fragments. Once tolerized, the patient would not develop an

autoimmune disease associated with the later appearance of the fragments in the patient. Tolerizing strategies involve purification of relevant autoantigenic fragments in a non-aggregated form. Low doses of the fragments are injected in pharmaceutically acceptable carriers, preferably without an adjuvant to induce low-zone tolerance.

The present invention also provides a method of generating an autoimmune response against certain cells in a patient. For example, if it is desirable to generate an autoimmune response against malignant cells in a patient, one can isolate a sample of the cells from the patient and contact the cells with the contents of granules isolated from granule containing lymphocytes. The action of the granule contents on the cells can produce autoantigenic fragments therefrom. In some embodiments, the malignant cells can be disrupted by lysis or mechanical breakage to release the contents of the cells before contacting the cells with the contents of the granules. In any case, the resulting mixture or purified components therefrom can be administered to the patient. Because the autoantigenic fragments produced in this way are the same as those produced *in vivo* by the action of granule containing lymphocytes, including *e.g.*, CTLs, NK and LAK cells, on the malignant cells, the immune system is thereby stimulated to generate a response against the malignant cells. Therefore, the present invention provides a method to heighten or stimulate the natural immune system processes to act against particular types of cells such as malignant cells.

The autoantigenic fragments useful in the invention described herein display cryptic epitopes. These epitopes are revealed to the immune system after cleavage of the precursor protein by the enzymes contained in granules to yield the autoantigenic fragments. There is a persuasive body of literature that reports that the highly specific humoral immune response to autoantigens in autoimmune disease is T cell-dependent, and that flares in autoimmune disease result when this primed immune system is rechallenged with a self-antigen (reviewed in Burlingame, R.W., *et al.*, 1993; Diamond, B., *et al.*, 1992; Radic, M.Z. and M. Weigert. 1994). However, the mechanisms responsible for initiation of the primary immune response to these molecules, and for subsequently stimulating the secondary response to

targeted antigens, have not been completely elucidated (Bach, J.F. and S. Koutouzov. 1997; Sercarz, E.E. and S.K. Datta. 1994). Several studies report that a potential for T cell autoreactivity resides in the immunological non-equivalency of different areas of self-molecules, since tolerance is only induced to dominant determinants which are generated and presented at suprathreshold concentrations during natural processing of whole protein antigens (reviewed in (Sercarz, E.E., *et al.*, 1993; Lanzavecchia, A. 1995). Those determinants which are not generated at all, or are generated at subthreshold levels during antigen processing (termed cryptic), do not tolerize T cells. Thus, potentially autoreactive T cells recognizing this cryptic self epitopeare allowed to persist.

The mechanisms through which autoimmunity can arise when normally cryptic determinants become visible to the immune system has received increased attention. Several experimental systems have now provided evidence that the balance of dominant versus cryptic epitopes in a self molecule can be profoundly influenced by forces which alter the 'immunological' structure of molecules (Lanzavecchia, A. 1995). Examples include the revelation of cryptic epitopes through novel cleavage (Bockenstedt, L.K., *et al.*, 1995; Mamula, M.J. 1993), or through altered conformation induced by high affinity ligand binding (e.g. to an antibody or receptor molecule (Salemi, S., *et al.*, 1995; Simitsek, P.D., *et al.*, 1995; Watts, C. and A. Lanzavecchia. 1993). The unique, high-titer autoantibody responses that characterize different autoimmune diseases can therefore be viewed as the immunologic impression of the initiating events that revealed suprathreshold concentrations of non-tolerized structure in a pro-immune context, thus satisfying the stringent criteria for initiation of a primary immune response (Casciola-Rosen, L. and A. Rosen. 1997).

The alteration of the structure of autoantigens during apoptosis is an important feature which underlies targeting of specific molecules by the immune system. Understanding whether and how the structure of those autoantigens are cleaved during apoptosis, provides insights into the role of apoptosis in initiation of the autoimmune response. One such mechanism involves the action of the enzymes in

contents of granules found in granule containing lymphocytes and is described herein. The contents of these granules are shown to act to reveal cryptic epitopes by cleaving autoantigens to autoantigenic fragments. In a preferred method, granzyme B cleaves autoantigenic proteins to create preferred autoantigenic fragments that display cryptic epitopes to the immune system.

Recent reports using caspase inhibitors have emphasized the contribution of caspase-independent pathway(s) when target cell death is induced by cytotoxic lymphocyte granule exocytosis (Sarin *et al.*, 1997; Talanian *et al.*, 1997). The results presented here demonstrate that several of the downstream substrates cleaved by the caspase family of proteases during apoptosis are also directly and efficiently cleaved by granzyme B both *in vitro* and in target cells undergoing lymphocyte granule-induced cytotoxicity. This confirms the existence of efficient caspase-independent proteolytic pathways during this form of cell death.

Not all downstream substrates of caspase-3 are cleaved by granzyme B with similar efficiency. In the case of PARP and U1-70kDa, cleavage efficiency by caspase-3 exceeds that by granzyme B by more than 200 fold, while NuMA and DNA-PK_{CS} are cleaved with similar efficiencies by granzyme B and caspase-3 (Table I). The relative cleavage efficiency of a given substrate by the two proteases appears to account for the results observed when caspase-3 activity is inhibited in intact cells. Thus, while PARP cleavage during granule-mediated cytotoxicity is inhibited by Ac-DEVD-CHO, cleavage of DNA-PK_{CS} and NuMA are only minimally affected. It is therefore believed that caspase-independent components of granule-mediated death are generated through altering the function of those downstream substrates that are efficiently cleaved by granzyme B (and potentially other granule proteases).

The results presented herein are consistent with recent studies in which granzyme B-induced proteolysis of PARP and U1-70kDa (both inefficient direct substrates for granzyme B) in an intact cell model was well inhibited by caspase inhibitors (Talanian *et al.*, 1997). The results differ from a previous study which failed to demonstrate unique DNA-PK_{CS} fragments during CTL-induced target cell death, or by granzyme B *in vitro* (Song *et al.*, 1996a). Different antibodies to DNA-

PK_{CS} used in the two studies may account for the failure of the previous study to find that a unique 100kDa C-terminal fragment is generated by granzyme B.

The results indicate that direct and efficient cleavage of NuMA and DNA-PK_{CS} by granzyme B achieves functional effects similar to those caused by caspase cleavage during other forms of apoptosis. This is supported by the demonstration that the cleavage of DNA-PK_{CS} by granzyme B completely abrogates its kinase activity (FIG. 3). This granzyme B-mediated cleavage of DNA-PK_{CS} differs from caspase-3-mediated cleavage of this substrate in several ways: (i) The presence of DNA ends renders the granzyme B-mediated cleavage significantly less efficient, while DNA ends are required for efficient cleavage of DNA-PK_{CS} by caspase-3 (Casciola-Rosen *et al.*, 1996; Song *et al.*, 1996b) (ii) While caspase-3 cleavage of DNA-PK_{CS} decreases its kinase activity to approximately 60% of control levels (Casciola-Rosen *et al.*, 1996), cleavage by granzyme B completely abrogates kinase activity. These data indicate that granzyme B cleaves and fully inactivates DNA-PK_{CS} very early during granule-mediated cytotoxicity, before significant internucleosomal DNA cleavage has occurred, and thus before caspase-3 can efficiently cleave DNA-PK_{CS}. Once abundant DNA ends have been generated, cleavage by caspase-3 should predominate (with the resulting fragments possessing residual kinase activity).

Although the functional consequences of substrate cleavage by caspases during apoptosis *in vivo* is not known for most substrates, recent reports demonstrate that cleavage can activate critical pro-apoptotic activities (e.g. activation of DNA fragmentation factor by caspase-3 (Liu *et al.*, 1997)). In other cases, reports suggest that substrate cleavage might disable important structural and homeostatic functions (Casciola-Rosen *et al.*, 1996; Ghayur *et al.*, 1996). It is therefore reported that the ability of cytolytic lymphocyte granule proteases to directly cleave some caspase substrates underlies the caspase-independent component of the death pathway induced by these cells (Sarin *et al.*, 1997). The ability of the lymphocyte granule-induced

cytotoxicity pathway to generate a novel form of nuclear condensation even in the absence of caspase activity, is consistent with this theory.

Studies on macromolecular and tetrapeptide substrates provide insights into the substrate specificity of granzyme B. For example, the direct cleavage of PARP by granzyme B occurs at $VGPD^{537}$-S^{538} (Froelich *et al.*, 1996a), while processing of several caspase precursors occurs at IETD sites (Fernandes-Alnemri *et al.*, 1996; Ramage *et al.*, 1995; Srinivasula *et al.*, 1996; Yamin *et al.*, 1996). The studies presented here demonstrate that granzyme B cleaves a macromolecular substrate (DNA-PK_{CS}) at VGPD-F^{2699}, a site consistent with the tetrapeptide substrate specificity of granzyme B defined using a combinatorial tetrapeptide library (Thornberry *et al.*, 1997). Interestingly, granzyme B also directly cleaves this substrate at a nearby DEVD-N^{2713}, suggesting that the determinants of macromolecular substrate specificity are more complex than those contained in the tetrapeptides tested.

Using the fragment sizes of NuMA and DNA-PK_{CS} generated by granzyme B, the known epitope specificity of several of the antibodies used, and the known substrate specificity of granzyme B, these studies indicate that the most likely granzyme B cleavage site generating the 100kDa C-terminal fragment of DNA-PK_{CS} is $VDQD^{3210}$-G^{3211}. Similarly, the most likely granzyme B cleavage site in NuMA occurs at $VLGD^{411}$-V^{412}. Several studies have addressed the cell biology of granzyme B during perforin/granzyme B-induced apoptosis (Froelich *et al.*, 1996b; Shi *et al.*, 1997; Trapani *et al.*, 1996). These studies reported that granzyme B autonomously enters the cytoplasm of target cells. That event alone does not induce target cell apoptosis. In the presence of perforin, however, apoptosis is induced in target cells. That event is accompanied by the rapid enrichment of granzyme B in nuclei and nucleoli of target cells (Jans *et al.*, 1996; Pinkoski *et al.*, 1996; Trapani *et al.*, 1996). It is therefore of importance that the granzyme B substrates described here are nuclear proteins. These substrates function in both structural and homeostatic pathways which are impacted by the caspases during apoptosis. The observation that a component of granule-induced cell death is caspase-independent, taken

together with the ability of caspases and granzyme B to efficiently cleave a common subset of downstream substrates at different sites during granule-induced cytotoxicity, highlights the importance of proteolysis of those common substrates in generating the apoptotic phenotype. Table II).

In the light of several descriptions of viral or endogenous caspase inhibitors (Beidler *et al.*, 1995; Bump *et al.*, 1995; Irmler *et al.*, 1997; Thome *et al.*, 1997; Xue and Horvitz, 1995), as well as long-lived cells or tumor cells which express low levels of specific caspase family members (Krajewska *et al.*, 1997; Krajewski *et al.*, 1997), the caspase-independent activity of granzyme B provides the host with apoptotic effector mechanisms that are insensitive to inhibitors of the signaling or execution components of the apoptotic cascade.

Many of the downstream caspase substrates described to date are autoantigens targeted in human systemic autoimmune diseases (Casciola-Rosen *et al.*, 1995; Casciola-Rosen *et al.*, 1994; Casciola-Rosen *et al.*, 1996; Casiano *et al.*, 1996; Greidinger *et al.*, 1996). The present demonstration that several of these substrates are also directly cleaved by granzyme B, generating unique fragments not generated during any other form of apoptosis studied to date, demonstrates how non-tolerized determinants of autoantigens can be revealed during certain forms of CTL-mediated apoptotic death.

In the experimental results presented herein, it is demonstrated that DNA-PK_{CS} and NuMA are directly cleaved by granzyme B, both *in vitro* and in cells undergoing granule-induced cytotoxicity. Although the efficiency of cleavage of these substrates is similar to those observed for caspase 3-mediated cleavage, the fragments generated by the 2 proteases are distinct. Since caspases appear to initiate apoptosis by altering the function of downstream substrates (either by decreasing the function of the intact substrate, or by generating fragment(s) with pro-apoptotic activity), it is believed that direct cleavage of caspase substrates by granzyme B during cytotoxic lymphocyte granule-induced apoptosis plays an important role in caspase-independent target cell death. The ability of the contents of cytotoxic lymphocyte granules to bypass the requirement for caspases in

the death pathway may guarantee the demise of target cells whose caspase pathway is incomplete or under strict endogenous or exogenous regulatory control.

The action of granzyme B in producing particular autoantigenic fragments from particular autoantigens is exemplified herein. However, the general understanding of the role of granule proteases as described and exemplified in the granzyme B model system allows one to generate these and other autoantigenic fragments. The autoantigenic fragments produced can be used in the preparation of pharmaceutical compositions, for treating patients at risk for or suffering from autoimmune diseases and cancer, and in assays for assessing the presence or absence of an autoimmune condition in a patient.

The following examples are presented by the way of illustration and, because various other embodiments will be apparent to those in the art, the following examples are not to be construed as a limitation on the scope of the invention.

EXAMPLE 1

General Materials and Methods

Materials. Purified DNA-dependent protein kinase (DNA-PK) and SP1 were purchased from Promega (Madison, WI). ATP was purchased from Fluka (Ronkonkoma, NY), and ^{32}P-ATP was from Du Pont/NEN (Wilmington, DE). Ac-DEVD-CHO and Ac-YVAD-CHO were manufactured by Merck (Rahway, NJ). Caspase-3 was purified as described (Nicholson *et al.*, 1995). Patient sera were used to immunoblot the nuclear mitotic apparatus protein (NuMA), poly(ADP-ribose) polymerase (PARP) and DNA-PK$_{cs}$ (Casciola-Rosen *et al.*, 1995; Greidinger *et al.*, 1996). Monoclonal antibodies can be made by methods known in the art. Two different monoclonal antibodies, designated 18-2 and 25-4 (kind gifts from Dr. Tim Carter, St. Johns University, Jamaica, NY) were also used to detect DNA-PK$_{cs}$ by immunoblotting (see Table II

for a summary of the antibodies used to detect DNA-PK_{cs} and its cleaved fragments). Rabbit polyclonal antibodies to caspases were raised against the large subunits of caspase-3 and caspase-7, using methods commonly known in the art. Immunoblotted proteins were detected using the SUPERSIGNAL™ substrate system (Pierce, Rockford, IL), according to the manufacturer's instructions.

In vitro cleavage of purified DNA-PK_{cs} and [^{35}S]methionine-labeled caspase-3 precursor, caspase-7 precursor, PARP, and NuMA. cDNAs for caspase-3, caspase-7, NuMA and PARP were used to drive the synthesis of [^{35}S]methionine-labeled proteins by coupled transcription/translation in rabbit reticulocyte lysates. For all purified substrates, cleavage reactions were performed in buffer consisting of 50 mM Hepes pH 7.4, 10% sucrose and 5 mM DTT in the presence of the granzyme B concentrations indicated in FIG. 1. After incubation at 37°C for 15 min, reactions were terminated and samples were electrophoresed on 10% (DNA-PK_{cs}, NuMA), 12% (PARP) or 15% (caspases 3 and 7) SDS-polyacrylamide gels. Radiolabeled proteins and their fragments were visualized by fluorography. Intact and cleaved DNA-PK_{cs} were visualized by immunoblotting with monoclonal antibody 18-2 (Casciola-Rosen *et al.*, 1995).

Calculation of catalytic constant values. Catalytic constant (k_{cat}/k_m) values were calculated essentially as described (Casciola-Rosen *et al.*, 1996). Briefly, subsaturating substrate concentrations were used in each *in vitro* reaction, and product appearance was assumed to be a first order process. Substrate and product bands on autoradiograms were scanned by densitometry. Several appropriate densitometry systems are available, *e.g.* PDI Discovery System, with Quantity One Software, Protein Databases, Inc., (Huntington Station, NY). kcat/Km values were calculated by fitting the dose-response data to the first order rate equation: percent substrate cleavage = $100*(1^{-e-((kcat*[E]/Km)*time)})$.

In vitro cleavage of endogenous DNA-PK_{cs}, NuMA, and PARP in HeLa lysates. Control HeLa lysates were prepared using methods commonly applied in the art, as described in Casciola-Rosen *et al.*, 1994. 12.5 nM purified granzyme B or 105 pM purified caspase-3

were then added to the lysates, in the absence or presence of 100nM Ac-DEVD-CHO or Ac-YVAD-CHO. The mixtures were incubated for 15 min on ice to facilitate binding of the inhibitors to proteases prior to performing cleavage reactions at 37°C for 60 min. After electrophoresing the samples on 10% SDS-polyacrylamide gels containing 0.087% bisacrylamide, the intact proteins and their cleaved fragments were visualized by immunoblotting.

Determination of granzyme B cleavage sites in DNA-PK_{CS} by P1 Asp mutagenesis. A partial cDNA clone for DNA-PK_{CS}, encoding Met^{2566} through Leu^{2928} (the region containing the caspase-3 cleavage site as well as the putative granzyme B site), was amplified by reverse-transcriptase PCR from HeLa cell poly(A)+ RNA using primers containing 5' EcoRI and 3' XbaI restriction enzyme-adapters. After ligation into the corresponding restriction sites of pBluescript II SK+ (Stratagene), this clone was used as template for mutagenesis by overlap-extension PCR to generate clones containing $D^{2698}A$ (P1 of the putative granzyme B site) and $D^{2712}A$ (P1 of the known caspase-3 site) modifications. [^{35}S]-Radiolabeled polypeptides were generated by coupled *in vitro* transcription/ translation, and then incubated with either recombinant caspase-3 (8 nM) or purified YT cell-derived granzyme B (8 nM) for 60 min at 37°C in a buffer composed of 50 mM Hepes/KOH (pH 7.0), 10% (w/v) sucrose, 2 mM EDTA, 0.1% (w/v) CHAPS, 5 mM dithiothreitol. The resulting cleavage products were resolved on SDS-polyacrylamide gels (10-20% gradient gels) and visualized by fluorography.

In vivo cleavage of endogenous DNA-PK_{CS}, NuMA and PARP during YT cell granule content-induced cytotoxicity of intact K562 cells. Intact cytoplasmic granules were purified from YT cells, and the granule contents were isolated using known methods (Tschopp, 1994). Some of these preparations were used for the further purification of granzyme B (Tschopp, 1994). Purity of the protease was confirmed by silver staining of overloaded SDS-polyacrylamide gels. The cytotoxic effects of YT granule contents were determined as follows: Jurkat T cells or K562 cells were radiolabeled with 100μCi/ml [^{51}Cr] sodium chromate,

washed, resuspended in Ca^{2+}-free HBSS, and then incubated with granule contents (0-5µl) and 1mM $CaCl_2$ for increasing times. The percentage specific [^{51}Cr] was calculated using the following formula: % specific lysis = [(sample cpm- spontaneous cpm)/(maximum cpm – spontaneous cpm)] x 100. *In vivo* experiments to assay the effect of YT cell granule contents on DNA-PK_{cs}, NuMA and PARP in intact K562 cells were performed as follows: K562 cells were washed twice with PD (2.7mM KCl, 1.5mM KH_2PO_4, 137mM NaCl, 8mM Na_2HPO_4), then resuspended at 1.7 x 10^7 cells/ml in PD in the absence or presence of 100µM Ac-DEVD-CHO, and incubated at 37°C for 30 min. Aliquots containing 3.4 x 10^5 cells were incubated for a further 90 min at 37°C with 1mM EDTA or 1mM Ca^{2+} and 2 µl of YT granule contents (which induces 20-40% specific chromium release in 60 min, and characteristic internucleosomal DNA degradation. The total reaction volume of each sample was 30µl. The reactions were terminated by boiling in SDS gel buffer and samples were electrophoresed and immunoblotted as described above. Experiments were performed using 2 different preparations of granule contents and 4 different cell types (K562, Jurkat, HeLa, primary human myoblasts).

Confocal Immunofluorescence microscopy. Morphologic experiments were performed on HeLa cells grown on No. 1 glass coverslips. Coverslips were washed three times with ice-cold HBSS without Ca^{2+}, prior to incubation (4°C, 30 min) with 25µl of HBSS minus Ca^{2+} containing 0.8µl of YT cell granule contents (see above), in the presence or absence of 200µM Ac-DEVD-CHO. 25µl of HBSS containing 2mM $CaCl_2$ was then added to each coverslip (mixed well by repeated, gentle aspiration), followed by incubation in a humidified chamber at 37°C for 60 min. The cells were then fixed in 4% paraformaldehyde (4°C, 5 min), permeabilized with acetone (4°C, 15 sec), and stained sequentially with antibodies to PARP or NuMA, propidium iodide and DAPI as described (Casciola-Rosen *et al.*, 1994a). Coverslips were mounted on glass slides with Permafluor (Lipshaw, Pittsburgh, PA), and confocal microscopy was performed on a scanning confocal microscopy system (LSM 410, Carl Zeiss, Inc., Thornwood, NJ).

LAK cell-mediated cytotoxicity. LAK cells were obtained by culturing human PBMCs for 4 days in LAK medium (RPMI supplemented with 10mM Hepes pH 7.4, L-glutamine, 2% autologous plasma), and 1000 Cetus units/ml of hrIL-2 (Chiron Therapeutics, Emeryville, CA) (Topalian *et al.*, 1989). Fas-negative target cells (K562 erythroleukemia cells) were resuspended at 1.3 x 10^6 cells/ml in LAK medium in the presence or absence of 100 μM Ac-DEVD-CHO, and incubated at 37°C for 60 min, prior to co-incubation with LAK effector cells (effector:target ratio of 5:1) for 4 h. After 2 washes with PD, cells were lysed and boiled in SDS sample buffer, and PARP, DNA-PK_{cs} and NuMA were assayed by immunoblotting as described above.

Kinase assay. Kinase assays were performed on intact DNA-PK_{cs} or DNA-PK_{cs} that had first been cleaved by granzyme B as follows. Reaction mixtures containing 10 mM Hepes pH 7.4, 2 mM $MgCl_2$, 10 mM KCl, 2.7 mM DTT, and 50 ng DNA-PK_{cs} in the absence or presence of 12.5 nM purified granzyme B, were incubated for 13.5 min at 37°C. Kinase reactions were subsequently initiated by adding 100 ng SP1 and 150 μM ATP containing 1.5 μCi [^{32}P]-ATP (3000Ci/mmol), in the absence or presence of 10 μg/ml sheared herring sperm DNA (Promega). Samples were incubated at 37°C for 10 min (well within the linear range of the assay, data not shown), before terminating the reactions by adding SDS gel buffer and boiling. After electrophoresing the samples on 8% SDS-PAGE, SP1 phosphorylation was detected by autoradiography, and quantitated by densitometry. Cleaved status of the kinase was confirmed in parallel by immunoblotting.

EXAMPLE 2

DNA-PK_{cs} and NuMA are very efficient substrates for purified granzyme B.

Granzyme B has previously been reported to cleave the precursors of several caspases (including caspases 3, 7 and 10), resulting in activation of their proteolytic activity. The catalytic efficiency of cleavage of these substrates by granzyme B serves as a useful

standard against which granzyme B-mediated cleavages of other substrates can be compared. Purified [^{35}S]methionine-labeled precursors of caspase-3 and caspase-7, or THP.1 cytosols (containing these precursor proteases) were incubated *in vitro* with increasing concentrations of purified granzyme B. The dose-response data obtained (FIG. 1) was used to calculate catalytic constant (k_{cat}/K_m) values of $1.8 \pm 0.6 \times 10^5 M^{-1}s^{-1}$ (radiolabeled substrate) and $1.9 \pm 0.1 \times 10^5 M^{-1}s^{-1}$ (immunoblotting) for caspase-7, and $3.6 \pm 1.0 \times 10^4 M^{-1}s^{-1}$ (radiolabeled substrate) and $2.3 \pm 0.4 \times 10^4 M^{-1}s^{-1}$ (immunoblotting) for caspase-3 (Table I). Thus, granzyme B cleaves caspase-7 approximately 6 fold more efficiently than caspase-3, consistent with previous reports (Talanian *et al.*, 1997).

It was then determined whether any of the downstream substrates known to be cleaved by caspases during apoptosis were also directly cleaved by granzyme B, and the efficiency of cleavage of each substrate by the two proteases was compared. Purified DNA-PK_{cs} was very efficiently cleaved by granzyme B in the absence of added DNA ($k_{cat}/K_m = 2.5 \pm 0.8 \times 10^6 M^{-1}s^{-1}$, see FIG. 1 and Table I), making it the best substrate for granzyme B described to date, with a cleavage efficiency two orders of magnitude better than that described for granzyme B-mediated cleavage of the caspase-3 precursor. When similar experiments were performed in the presence of 10μg/ml DNA, DNA-PK_{cs} cleavage was decreased by approximately 90% .

Caspase-3-mediated cleavage of DNA-PK_{cs} was also extremely efficient (k_{cat}/K_m value = $7.5 \pm 0.8 \times 10^6 M^{-1}s^{-1}$). In this case, efficient cleavage was only obtained in the presence of DNA ((Casciola-Rosen *et al.*, 1995), and Table I). NuMA, a nuclear matrix protein that is cleaved in apoptotic cells by an unidentified protease with features of the caspase family, was also very efficiently cleaved by granzyme B (k_{cat}/K_m value = $5.4 \pm 1.4 \times 10^5 M^{-1}s^{-1}$, see Table I). The efficiency of this cleavage was one order of magnitude greater than that observed for granzyme B-mediated processing of the caspase-3 precursor. NuMA was also efficiently cleaved by purified caspase-3, with a k_{cat}/K_m value of = $5.0 \pm 1.0 \times 10^5 M^{-1}s^{-1}$. (Table I). In contrast to DNA-PK_{cs} and NuMA (where granzyme B- and caspase-3-mediated cleavages are similarly efficient),

PARP was a relatively poor substrate for granzyme B, with a k_{cat}/K_m value ($2.3 \pm 1.8 \times 10^4\ M^{-1}s^{-1}$) that is approximately 200 fold lower than that for caspase-3 (see Table I). Granzyme B was also a poor catalyst for cleaving U1-70kDa, with k_{cat}/K_m values $<10^3\ M^{-1}s^{-1}$. The efficiency of substrate cleavage by granzyme B is therefore similar to caspase-3 for some substrates (*e.g.* DNA-PK_{cs} and NuMA), while it is more than 2 orders of magnitude less efficient for others (*e.g.* PARP and U1-70kDa).

EXAMPLE 3

Different substrate fragments are detected after cleaving autoantigens *in vitro* with granzyme B or caspase-3.

To directly compare the fragments generated by granzyme B and caspase-3, purified DNA-PK_{cs}, *in vitro* translated [^{35}S]methionine-labeled PARP, and endogenous substrates (NuMA and DNA-PK_{cs} in HeLa cell lysates) were incubated with protease and electrophoresed in adjacent lanes. When granzyme B was used to cleave DNA-PK_{cs}, fragments of 100kDa and 250kDa were generated, (detected by immunoblotting using antibodies recognizing the C-terminus or N-terminus of DNA-PKcs, respectively) (FIG. 2, lanes 2 & 5; and Table II). In contrast, caspase-3 cleavage yielded a 150kDa C-terminal fragment (FIG. 2, lane 3) and a 250kDa N-terminal fragment (FIG. 2, lane 6).

Granzyme B-mediated cleavage of NuMA generated a novel fragment migrating at 175kDa on SDS-PAGE, which was distinct from the 185kDa fragment detected after cleavage with caspase-3 (FIG. 2, lanes 7-9). Similarly, novel fragments of PARP migrating at 72, 62 and 42kDa were detected after incubation with granzyme B; these differed from the 89 and 24 kDa fragments generated by caspase-3-mediated cleavage of PARP (FIG. 2, lanes 10-12 and Table II). Granzyme B therefore directly cleaves several of the downstream substrates of caspase-3 *in vitro*. In all cases, the fragments generated by granzyme B differ from those generated by caspase-3.

EXAMPLE 4

Kinase activity of DNA-PK$_{CS}$ is abolished by granzyme B cleavage.

To determine the effect of granzyme B-mediated cleavage on the kinase activity of DNA-PK$_{CS}$, the ability of intact and cleaved DNA-PK$_{CS}$ to phosphorylate the SP1 transcription factor was quantitated. The kinase activity of DNA-PK$_{CS}$ was entirely DNA-dependent (FIG. 3, lanes 1 and 2). When DNA-PK$_{CS}$ was pretreated with 12.5nM granzyme B for 13.5 min at 37°C (which cleaves DNA-PK$_{CS}$ (FIG. 1)), followed by the addition of DNA, [^{32}P]ATP and SP1 (which initiate the phosphorylation reaction), kinase activity was entirely abolished (FIG. 3, lane 3). Whether any of the fragments generated by granzyme B-mediated cleavage of DNA-PK$_{CS}$ have novel activity(ies) in addition to the autoantigenic reactions demonstrated herein remains to be determined.

EXAMPLE 5

Granzyme B induces novel fragments of DNA-PK$_{CS}$ and NuMA in cell lysates.

Previous studies reported that the cleavage of PARP that occurs in lysates of COS cells expressing granzyme B can be almost completely inhibited by the caspase inhibitor, Ac-DEVD-CHO (Darmon *et al.*, 1995). Because this compound is not an inhibitor of granzyme B, this observation suggests that this cleavage is mediated solely by caspases which have been activated by granzyme B in these extracts. The observation that PARP itself is cleaved by granzyme B approximately 200 fold less efficiently than by caspase-3 further supports this conclusion (see Table I). Since NuMA and DNA-PK$_{CS}$ are cleaved with similar efficiencies by caspase-3 and granzyme B, it was of interest to determine whether these substrates are cleaved directly by granzyme B in cell lysates that contain caspase precursors. Therefore, an *in vitro* assay system was established in which cleavage of endogenous DNA-PK$_{CS}$, NuMA or PARP was monitored after addition of purified caspase-3 or

granzyme B. The assay was conducted in the presence or absence of the caspase inhibitor, Ac-DEVD-CHO.

Incubation of control extracts at 37°C for 60 minutes resulted in no significant cleavage of DNA-PK_{CS} or PARP, but was associated with minimal cleavage of NuMA, leading to the production of minor fragments of 185 and 187kDa (FIG. 4, lane 1). When purified caspase-3 was added to control extracts, caspase-3-specific fragments of PARP (89kDa), NuMA (185kDa) and DNA-PK_{CS} (250kDa N-terminal fragment, 150kDa C-terminal fragment) were generated (FIG. 4, lane 2). As demonstrated above, substrate fragments generated by the activity of caspase-3 are entirely abolished by 100nM Ac-DEVD-CHO (K_i caspase-1 = 17nM; K_i caspase-3 = 0.2nM; K_i caspase-6 = 31nM; K_i caspase-7 = 1nM; K_i caspase-8 = 0.92nM ; K_i caspase-9 = 60nM; K_i caspase-10 = 12nM) (FIG. 4, lane 3), but were unaffected by 100nM Ac-YVAD-CHO (K_i caspase-1 = 0.6nM; K_i caspase-3 and caspase-7 >10μM; K_i caspase-10 = 408nM) (FIG. 4, lane 4).

In the presence of 12.5nM granzyme B, all three substrates were efficiently cleaved (FIG. 4, lane 5). The predominant PARP fragment induced by granzyme B co-migrated with the fragment induced by caspase-3 (FIG. 4, lane 5). There was also a minor (<5%) fragment of 62kDa which corresponded with the fragment induced by granzyme B on purified substrate. In contrast, only a minor proportion of the NuMA fragments induced by granzyme B co-migrated with the fragment generated by caspase-3 (185kDa). The major, novel fragment of 175kDa (FIG. 4, lane 5) corresponded with the fragment induced by granzyme B on purified *in vitro*-translated substrate. Granzyme B-mediated cleavage of DNA-PK_{CS} generated a 250kDa N-terminal fragment co-migrating with that generated by caspase-3 (FIG. 4, lanes 2 & 5), as well as a unique 100kDa C-terminal fragment which corresponded with that induced by granzyme B on purified substrate (FIG.2, lane 2).

When Ac-DEVD-CHO or 2mM iodoacetamide were added to the extracts 15 minutes prior to the addition of granzyme B, cleavage of PARP was almost entirely abolished (FIG. 4, lane 6). In contrast, the cleavage of NuMA and DNA-PK_{CS} was only partially inhibited (FIG. 4,

lane 6) (20-40% inhibition in 5 separate experiments). Generation of caspase-3-specific fragments of both NuMA (185kDa) and DNA-PKcs (150kDa C-terminal fragment was abolished under these circumstances, while formation of the 250kDa N-terminal fragment of DNA-PK_{CS} was inhibited by 20-40% (FIG. 4, lane 6).

Generation of granzyme B-specific fragments (175kDa NuMA fragment and 100kDa C-terminal DNA-PK_{CS} fragment) was not affected by the inhibitors of caspase (FIG. 4, lane 6). The failure of Ac-DEVD-CHO to markedly inhibit generation of the 250kDa DNA-PK_{CS} fragment indicates that the majority of DNA-PK_{CS} cleavage detected after addition of granzyme B to lysates results from direct cleavage by this protease (rather than indirectly through activation of caspase-3).

Taken together, the results demonstrate that granzyme B competes with caspase-3 for cleavage of endogenous substrates in cell lysates. The outcome of this competition can be accurately predicted by comparing k_{cat}/K_m values: for those substrates where k_{cat}/K_m for cleavage by caspase-3 is greater than that of granzyme B *(e.g.* PARP), caspase-3 fragments are detected almost exclusively. For those substrates where the k_{cat}/K_m values are similar for the proteases, addition of granzyme B results in the formation of novel, granzyme B-specific fragments *(e.g.* DNA-PK_{CS} and NuMA).

EXAMPLE 6

<u>Granzyme B cleavage sites in DNA-PK_{CS}</u>

To address whether the 250kDa DNA-PK_{CS} fragment generated by caspase-3 and granzyme B result from cleavage at the same and/or different, but closely-spaced sites, not distinguishable by SDS-PAGE, we generated a fragment of DNA-PK_{CS} (Met^{2566} - Leu^{2928}) encompassing both the known caspase-3 cleavage site at $DEVD^{2712}$-N^{2713} (Casciola-Rosen *et al.*, 1996; Song *et al.*, 1996b), as well as the potential granzyme B cleavage site at $VGPD^{2698}$-F^{2699}. The relevant P_1 aspartic acids (D^{2698} and D^{2712}) in this fragment were mutated to alanines ($D^{2698}A$; $D^{2712}A$), and susceptibility of wild type and mutated

forms to cleavage by caspase-3 or granzyme B was assessed (FIG. 5). Cleavage of the wild type protein by caspase-3 resulted in two fragments of 26kDa and 20kDa (FIG. 5, lanes 1,2); this cleavage was entirely abolished by the $D^{2712}A$ mutation (FIG. 5, lanes 7,8), confirming that caspase-3 cleaves at $DEVD^{2712}$-N^{2713}. In addition to 20kDa and 26kDa fragments identical to those generated by caspase-3, granzyme B cleavage also resulted in fragments of 28kDa and 18kDa (FIG. 5, lane 3); these unique fragments were enhanced by the $D^{2712}A$ mutation (FIG. 5, lane 9), but were abolished by the $D^{2698}A$ mutation (FIG. 5, lane 6), placing a granzyme B cleavage site at $VGPD^{2698}$-F^{2699}. These data demonstrate that granzyme B can cleave at $VGPD^{2698}$-F^{2699}, a site predicted by previous studies using a combinatorial tetrapeptide substrate library (Thornberry *et al.*, 1997). Furthermore, granzyme B also cleaves at the caspase-3 cleavage site, $DEVD^{2712}$-N^{2713}. The C-terminal granzyme B-unique cleavage site is believed to be $VDQD^{3210}$-G^{3211}. This cleavage yields the approximately 100 kDa fragment from amino acids 3212 to 4096.

EXAMPLE 7

<u>Granzyme B-specific fragments of DNA-PK_{CS} and NuMA are generated during cytotoxic lymphocyte granule-induced target cell death.</u>

To determine whether the granzyme B-specific fragments demonstrated in cell lysates also occur when target cell death is induced by the contents of cytolytic lymphocyte granules, The consequences of granule-induced target cell death on substrate cleavage were examined. Granules containing perforin and granzymes were purified from the human NK cell line, YT. The granule contents were harvested and used to induce target cell cytotoxicity as described in Example 1. Several different target cells (Jurkat T cells, K562 erythroleukemia cells, human myoblasts or HeLa cells) were incubated with granule contents (~1.5 x 10^7 YT cell equivalents/ml) in the presence of Ca^{2+}. Rapid target cell lysis was induced (achieving ~20%-40% specific ^{51}Cr release in 60 min). Target cell lysis by granule contents did not occur in the absence of Ca^{2+}

(Podack and Konigsberg, 1984; Young *et al.*, 1986). DNA-PK_{CS}, NuMA and PARP were all cleaved rapidly after addition of granules, in a Ca^{2+}-dependent manner (FIG. 6, lanes 2 & 3). As observed in the lysate system described above, cleavage of PARP resulted almost completely in the generation of the 89kDa caspase-3-specific fragment (FIG. 6, lane 3). Only small amounts of the 62kDa granzyme B-specific fragment were generated. Generation of the 89kDa fragment was completely inhibited by Ac-DEVD-CHO (FIG. 6, lane 4). In contrast, granule contents induced the formation of granzyme B-specific cleavage fragments of DNA-PK_{CS} and NuMA (FIG. 6, lane 3). The production of these fragments was not inhibited by Ac-DEVD-CHO (FIG. 6, lane 4).

Note that in the case of DNA-PK_{CS}, addition of granule contents also resulted in the formation of small amounts of 150kDa and 120kDa fragments, which were only well visualized after longer exposures of the X-ray film. Generation of these fragments was also entirely inhibited by Ac-DEVD-CHO.

EXAMPLE 8

<u>Granzyme B-specific fragments of DNA-PK_{CS} and NuMA are generated in Fas-negative target cells attacked by lymphokine-activated killer (LAK) cells.</u>

To determine whether granzyme B-specific fragments were generated during lymphocyte-induced cytotoxicity, we used the Fas-negative cell line K562 as targets for LAK cells (McGahon *et al.* 1995; Topalian *et al.*, 1989). To permit biochemical analysis of cleaved proteins in target cells, effector:target cell ratios of 5:1 were used. The signature 89kDa caspase-3 fragment of PARP was generated during LAK-induced target cell death (FIG. 7, lane 3). PARP cleavage was entirely abolished by 100μM Ac-DEVD-CHO (FIG. 7, lane 4). This was consistent with results observed when target cell death was initiated with YT cell granule contents (FIG. 6, lane 4). Using anti-C-terminal antibodies, generation of both the 100kDa granzyme B-specific fragment of DNA-

PK_{CS} and the 150-kDa caspase-3-specific fragment were detected during LAK-induced target cell death (FIG. 7, lane 3). In addition, a 120kDa fragment was observed in these cells, consistent with the caspase-3-mediated cleavage of DNA-PK_{CS} at DWVD-G previously observed both *in vitro* and in intact cells (Casciola-Rosen *et al.*, 1996; Song *et al.*, 1996b). While 100μM Ac-DEVD-CHO inhibited the generation of caspase-3-specific fragments of DNA-PK_{CS} by >90%, the 100kDa granzyme B-specific fragment was insensitive to this inhibitor (FIG. 7, lane 4). This data is consistent with that obtained after initiating target cell death with YT cell granule contents (FIG. 6, lane 4 and data not shown). Similar observations were made for the granzyme B-specific NuMA fragment. Together, these data confirm that the novel fragments of DNA-PK_{CS} and NuMA defined in these studies are indeed generated when intact lymphocytes induce target cell cytotoxicity using the granule pathway.

EXAMPLE 9

<u>Cytotoxic lymphocyte granules induce nuclear morphologic changes: Effects of caspase inhibitors.</u>

Several of the downstream substrates for the caspases are directly and efficiently cleaved by granzyme B during lymphocyte granule-induced cytotoxicity even in the presence of caspase inhibitors. It was determined whether these granules also induced morphologic changes in the target cell. HeLa cells were pre-incubated with YT cell granule contents in the presence or absence of 100μM Ac-DEVD-CHO, prior to addition of Ca^{2+}, and further incubation. YT cell granule contents induced the rapid onset (<60 min) of prominent surface blebbing (FIG. 8A), followed by nuclear condensation and fragmentation into membrane-bound apoptotic bodies (FIG. 8B). As described for UVB-induced apoptosis (Casciola-Rosen *et al.* 1994), the autoantigens targeted in systemic autoimmune diseases are rapidly redistributed in target cells exposed to YT cell granule contents, such that they become

clustered around the rim of the condensing apoptotic nucleus (FIG. 8A), and then ultimately around apoptotic bodies (FIG. 8B). Granule content-induced surface blebbing, nuclear fragmentation, formation of apoptotic bodies, and characteristic redistribution of nuclear autoantigens was prevented by Ac-DEVD-CHO (Compare FIG. 8B, 8C). However, a prominent diminution in the size of the nucleus (which was accompanied by condensation of chromatin) was induced by granule contents in Ac-DEVD-CHO-treated cells (FIG. 8C); these nuclear changes were not observed when cells were incubated with Ac-DEVD-CHO alone.

EXAMPLE 10

Antibodies Against Autoantigens and Autoantigenic Fragments.

The present invention also relates to polyclonal and monoclonal antibodies raised in response to the autoantigenic fragments disclosed herein. An antibody is specific for an epitope of an autoantigenic fragment if one of skill in the art can use standard techniques to determine conditions under which one can detect an autoantigenic fragment in a Western Blot of a sample from cells of a tissue. The blot can be of a native or denaturing gel as appropriate for the epitope. An antibody is highly specific for an autoantigenic fragment epitope if no nonspecific background binding is visually detectable. An antibody can also be considered highly specific for an autoantigenic fragment if the binding of the antibody can not be competed by random peptides, polypeptides or proteins, but can be competed by the particular autoantigenic fragment, autoantigen, or peptides or polypeptides derived therefrom.

Autoantigenic fragments can be separated from other cellular proteins by use of an immunoaffinity column made with monoclonal or polyclonal antibodies specific for the autoantigen. Additionally, polyclonal or monoclonal antibodies can be raised against a synthetic peptide (usually from about 9 to about 25 amino acids in length) from a portion of an autoantigen or autoantigenic fragment.

Monospecific antibodies are purified from mammalian antisera containing antibodies reactive against the autoantigenic fragment or are prepared as monoclonal antibodies using the technique of Kohler and Milstein (1975, *Nature* 256: 495-497). Monospecific antibody as used herein is defined as a single antibody species or multiple antibody species with homogenous binding characteristics for the autoantigenic fragment. Homogenous binding as used herein refers to the ability of the antibody species to bind to a specific antigen or epitope, such as those associated with the autoantigenic fragment, as described herein. Autoantigenic fragment -specific antibodies are raised by immunizing animals such as mice, rats, guinea pigs, rabbits, goats, horses and the like, with an appropriate concentration of autoantigenic fragment or a synthetic peptide generated from a portion of the autoantigenic fragment with or without an immune adjuvant.

Preimmune serum is collected prior to the first immunization. Each animal receives between about 0.1 mg and about 1000 mg of autoantigenic fragment associated with an acceptable immune adjuvant. Such acceptable adjuvants include, but are not limited to, Freund's complete, Freund's incomplete, alum-precipitate, water in oil emulsion containing *Corynebacterium parvum* and RNA. The initial immunization consists of injecting autoantigenic fragment or peptide fragment thereof, preferably in Freund's complete adjuvant, at multiple sites either subcutaneously (SC), intraperitoneally (IP) or both. Each animal is bled at regular intervals, preferably weekly, to determine antibody titer. The animals may or may not receive booster injections following the initial immunization. Those animals receiving booster injections are generally given an equal amount of autoantigenic fragment in Freund's incomplete adjuvant by the same route. Booster injections are given at about three week intervals until maximal titers are obtained. At about 7 days after each booster immunization or about weekly after a single immunization, the animals are bled, the serum collected, and aliquots are stored at about -20°C.

Monoclonal antibodies (mAb) reactive with the autoantigenic fragment are prepared by immunizing inbred mice, preferably Balb/c, with the autoantigenic fragment. The mice are

immunized by the IP or SC route with about 1 mg to about 100 mg, preferably about 10 mg, of the autoantigenic fragment in about 0.5 ml buffer or saline incorporated in an equal volume of an acceptable adjuvant, as discussed herein. Freund's complete adjuvant is preferred. The mice receive an initial immunization on day 0 and are rested for about 3 to about 30 weeks. Immunized mice are given one or more booster immunizations of about 1 to about 100 mg of the autoantigenic fragment in a buffer solution such as phosphate buffered saline by the intravenous (IV) route. Lymphocytes, from antibody positive mice, preferably splenic lymphocytes, are obtained by removing spleens from immunized mice by standard procedures known in the art. Hybridoma cells are produced by mixing the splenic lymphocytes with an appropriate fusion partner, preferably myeloma cells, under conditions which will allow the formation of stable hybridomas. Fusion partners can include, but are not limited to: mouse myelomas P3/NS1/Ag 4-1; MPC-11; S-194 and Sp 2/0, with Sp 2/0 being preferred. The antibody producing cells and myeloma cells are fused in polyethylene glycol, about 1000 mol. wt., at concentrations from about 30% to about 50%. Fused hybridoma cells are selected by growth in hypoxanthine, thymidine and aminopterin supplemented Dulbecco's Modified Eagles Medium (DMEM) by procedures known in the art. Supernatant fluids are collected form growth positive wells on about days 14, 18, and 21 and are screened for antibody production by an immunoassay such as solid phase immunoradioassay (SPIRA) using the autoantigenic fragment as the antigen. The culture fluids are also tested in the Ouchterlony precipitation assay to determine the isotype of the mAb. Hybridoma cells from antibody positive wells are cloned by a technique such as the soft agar technique of MacPherson, 1973, Soft Agar Techniques, in *Tissue Culture Methods and Applications*, Kruse and Paterson, Eds., Academic Press.

Monoclonal antibodies are produced *in vivo* by injection of pristine primed Balb/c mice, approximately 0.5 ml per mouse, with about 2×10^5 to about 6×10^6 hybridoma cells about 4 days after priming. Ascites fluid is collected at approximately 8-12 days after cell transfer

and the monoclonal antibodies are purified by techniques known in the art.

In vitro production of anti-autoantigenic fragment mAb is carried out by growing the hybridoma in DMEM containing about 2% fetal calf serum to obtain sufficient quantities of the specific mAb. The mAb are purified by techniques known in the art.

Antibody titers of ascites or hybridoma culture fluids are determined by various serological or immunological assays which include, but are not limited to, precipitation, passive agglutination, enzyme-linked immunosorbent antibody (ELISA) technique and radioimmunoassay (RIA) techniques. Similar assays are used to detect the presence of the autoantigenic fragment in body fluids or tissue and cell extracts.

It is readily apparent to those skilled in the art that the herein described methods for producing monospecific antibodies can be utilized to produce antibodies specific for autoantigenic fragment peptide fragments, or full-length autoantigen.

Antibody affinity columns are made, for example, by adding the antibodies to Affigel-10 (Biorad), a gel support which is pre-activated with N-hydroxysuccinimide esters such that the antibodies form covalent linkages with the agarose gel bead support. The antibodies are then coupled to the gel via amide bonds with the spacer arm. The remaining activated esters are then quenched with 1M ethanolamine HCl (pH 8). The column is washed with water followed by 0.23 M glycine HCl (pH 2.6) to remove any non-conjugated antibody or extraneous protein. The column is then equilibrated in phosphate buffered saline (pH 7.3) and the cell culture supernatants or cell extracts containing the autoantigenic fragment are slowly passed through the column. The column is then washed with phosphate buffered saline until the optical density (A_{280}) falls to background, then the protein is eluted with 0.23 M glycine-HCl (pH 2.6). The purified autoantigenic fragment is then dialyzed against phosphate buffered saline.

Levels of an autoantigenic fragment in cells and tissues is quantified by a variety of techniques including, but not limited to, immunoaffinity and/or ligand affinity techniques. Autoantigenic

fragment affinity beads or autoantigenic fragment-specific antibodies are used to isolate ^{35}S-methionine labeled or unlabelled autoantigenic fragment. Labeled autoantigenic fragment is analyzed by SDS-PAGE. Unlabelled autoantigenic fragment is detected by Western blotting, ELISA or RIA assays employing either autoantigenic fragment specific antibodies and/or antiphosphotyrosine antibodies.

Preferred antibodies that bind to an autoantigenic fragment but do not bind to the intact autoantigen or other fragments thereof. Examples of preferred antibodies are those that recognizes a cryptic epitope revealed in the autoantigenic fragment, or an antibody that recognizes a terminal epitope present only in the autoantigenic fragment.

EXAMPLE 11

Assay for the Detection of an Autoimmune Condition.

The autoantigenic fragments produced and identified following the teaching of the present invention can be used in a assay to detect the presence of an autoimmune condition. The condition can be the generation of autoantigenic fragments before a disease state evolves, the presence of an autoimmune disease or the lessening of a disease.

The assay is performed on a sample derived from a patient. Most commonly, the sample will be a tissue sample. The presence of autoantigenic fragments can be detected *in situ* or can be partially purified before conducting the assay.

To perform an assay within this invention one prepares an autoantigenic fragment. For example, one can prepare an autoantigenic fragment of DNA PK_{CS} by cleaving the protein with granzyme B. The autoantigenic fragment is then used to prepare a monoclonal or polyclonal antibody using any of the methods widely known and used in the art.

The antibody can then be used to qualify or quantify the amount of autoantigenic fragment present in the sample. This can be done by numerous techniques known in the art including using antibody

detectably labeled with ^{125}I, gold, enzyme or other known labels. Alternatively, a detectable label can be carried on a second antibody specific for the first. The amount of autoantigenic fragment found is quantitatively or qualitatively compared to the amount of found on control cells. A change in the former relative to the latter is indicative of whether an autoimmune disease state is present, is progressing or is reduced.

In an alternative form of the assay one can treat cells as described herein and then isolate the autoantigenic fragments present in treated and control cells. The preparations can be made as crude cell extracts, membrane or intracellular fractions of the cells or after purification steps, *e.g.*, chromatography, precipitation or affinity isolation steps. Crude, partially or highly purified preparations can be analyzed for autoantigenic fragment content, *e.g.*, by using antibodies specific for the autoantigenic fragment.

In another form of the assay, an autoantigenic fragment is used to determine the presence or absence of an autoantibody in a patient as an indication of the presence or absence of an autoimmune condition. The use of particular types or autoantigenic fragments can also indicate the type of autoimmune condition. The autoantibody to be assayed for can be present in the serum or a tissue sample of the patient. An autoantibody can be detected *in situ* or after some purification of immunoglobins from the patient. In one format of the assay, the autoantigenic fragment can be fixed to a support, an autoantibody present in a sample is then contacted with the fragment to permit binding of the autoantibody to the autoantigenic fragment. After appropriate washing, the presence of bound autoantibody can be detected by methods available in the art, including the use of a labeled second antibody against the antibodies from the patient.

In any assay it can be advantageous to devise an internal control so that the results of different runs of assays can be compared to each other. A cellular protein that is unrelated to the autoantigenic fragment and present in relatively constant amounts in the cells used in the assay can serve as an internal control.

The assays described above are exemplary of all of the assays within the scope of the present invention. Those of skill in the art can use the autoantigenic fragments and antibodies of this invention in many assay formats known or developed in the art.

EXAMPLE 12

Tolerizing a Patient to the Presence of an Autoantigenic fragment.

The present invention provides a method of tolerizing a patient to the future *in vivo* generation of compounds that are normally autoantigenic. This method can be prophylactic.

A patient diagnosed to be at risk of developing an autoimmune response is identified. A sample of the tissue to which the autoimmune response is possible is isolated from the patient. Autoantigenic fragments that can be generated from the tissue are then identified. The autoantigenic fragments are administered to the patient in pharmaceutically acceptable carriers without an adjuvant to induce low-zone tolerance.

Tolerization typically involves purification of relevant autoantigenic fragments in a non-aggregated form. In particular embodiments, autoantigenic fragments of DNA Pk_{CS}, NuMA or PARP are generated by the action of granzyme B.

The autoantigenic fragments can also be present in a mixture. One such mixture can be the product of the application of the contents of granules to a sample of tissue to which a potential autoimmune response is diagnosed. In that case, the autoantigenic fragments are produced in the mixture by the action of the granule contents, including granzyme B.

In any case, the autoantigenic fragments are administered at a low dose as chosen by a skilled physician or veterinarian to induce a low-zone tolerance in the patient. Once tolerization of the patient is achieved, if the normally autoantigenic fragments are produced in the tissue *in vivo*, the immune system will not mount a response against

them and the occurrence of an autoimmune disease state can be avoided or the severity reduced.

EXAMPLE 13

Treatment for Malignant Cells.

The present invention also provides a method of generating an autoimmune response against certain cells in a patient. For example, one can induce an autoimmune response against malignant cells in a patient that would benefit from such a response.

For example, one can isolate a sample of malignant cells from a patient and contact the cells with the contents of granules or granzyme B. The action of the granule contents or granzyme B on the cells can produce autoantigenic fragments from autoantigens present in the cells. The resulting mixture can then be administered to the patient. In this case, it is preferred that an adjuvant be administered with the autoantigenic fragments.

Those components of the mixture that are not altered to produce a new antigen will be recognized by the immune system as self molecules. However, those components that are altered to produce new autoantigenic fragments will be seen by the immune system as non-self and an immune response will be generated against them.

Because the autoantigenic fragments produced in this way are the same as those produced *in vivo* by the action of CTLs, NK and LAK cells on the malignant cells, the immune system is stimulated to generate a response against the malignant cells. Therefore, the present invention provides a method to heighten or stimulate the natural immune system processes to act against particular types of cells such as malignant cells. The method is particularly advantageous because the *in vivo* production of the autoantigenic fragments from, *e.g.*, malignant cells, can occur at rates too low to stimulate the immune system, or at rates that can lead to a tolerization of the immune system.

EXAMPLE 14

Pharmaceutical Compositions.

Pharmaceutically useful compositions comprising autoantigenic fragments of the present invention can be formulated according to known methods such as by the admixture of a pharmaceutically acceptable carrier. Examples of such carriers and methods of formulation can be found in Remington's Pharmaceutical Sciences. To form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the inhibitor.

Therapeutic, prophylactic or diagnostic compositions of the invention are administered to an individual in amounts sufficient to treat or diagnose disorders. The effective amount can vary according to a variety of factors such as the individual's condition, weight, sex and age. Other factors include the mode of administration. The appropriate amount can be determined by a skilled physician

The pharmaceutical compositions can be provided to the individual by a variety of routes such as subcutaneous, topical, oral and intramuscular.

The term "chemical derivative" describes a molecule that contains additional chemical moieties which are not normally a part of the base molecule. Such moieties can improve the solubility, half-life, absorption, etc. of the base molecule. Alternatively the moieties can attenuate undesirable side effects of the base molecule or decrease the toxicity of the base molecule. Examples of such moieties are described in a variety of texts, such as Remington's Pharmaceutical Sciences.

Compositions including autoantigenic fragments identified according to the methods disclosed herein can be used alone at appropriate dosages. Alternatively, co-administration or sequential administration of other agents can be desirable.

The present invention also provides a means to obtain suitable topical, oral, systemic and parenteral pharmaceutical formulations for use in the methods of treatment of the present

invention. The compositions containing autoantigenic fragments identified according to this invention as the active ingredient can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compositions can be administered in such oral dosage forms as tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection, as appropriate. Likewise, they can also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts.

Advantageously, autoantigenic fragments of the present invention can be administered in a single daily dose, or the total daily dosage can be administered in divided doses of two, three or four times daily.

Furthermore, compounds for the present invention can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

For combination treatment with more than one active agent, where the active agents are in separate dosage formulations, the active agents can be administered concurrently, or they each can be administered at separately staggered times.

The dosage regimen utilizing compositions of the present invention is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; whether the treatment is prophylactic or therapeutic; the severity of the condition to be treated; the route of administration; the renal, hepatic and cardiovascular function of the patient; and the particular compound thereof employed. A physician or veterinarian of ordinary skill can readily determine and prescribe the effective amount of the drug

required to prevent, counter or arrest the progress of the condition. Optimal precision in achieving concentrations of compounds of this invention, including purified autoantigenic fragments, within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the compound's availability to target sites. This involves a consideration of the distribution, equilibrium, and elimination of a compound.

EXAMPLE 15

Autoantigens Cleaved by Granzyme B

Granzyme B efficiently cleaves three caspase-3 substrates generating unique fragments not generated during any other form of cell death. To determine whether the generation of unique autoantigen fragments by granzyme B was a universal feature of autoantigens, a wide range of autoantigens were tested for cleavage by granzyme B *in vitro* and *in vivo*. It was determined that despite their diverse structure, distribution and function, >70% of the autoantigens described in systemic autoimmune diseases are efficiently cleaved by granzyme B and unique fragments are produced. In contrast, granzyme B does not generate unique fragments in all the non-autoantigen molecules tested. A panel of autoantigens discovered to be susceptible to cleavage by granzyme B, are listed in Table 3 along with the sites of cleavage.

The granzyme B cleavage sites in autoantigens were defined. In all cases, the tetrapeptide sequence immediately adjacent to the cleavage site was highly conserved. The susceptibility to granzyme B cleavage is therefore a specific, unifying feature of these otherwise unrelated molecules. Furthermore, the ability of granzyme B to generate unique fragments of these antigens indicates that granzyme B plays a mechanistic role in selectively producing the fragments of these molecules against which autoimmune responses are initiated. These results highlight a potential role for the cytotoxic lymphocyte granule-induced death pathway in initiation and propagation of autoimmunity.

To determine whether susceptibility to direct cleavage by granzyme B was an isolated feature of PARP, NuMA and DNA-PK_{cs} shown above, or whether it was a more general feature of autoantigens, a variety of well-defined autoantigens selected from across the spectrum of systemic autoimmune diseases were tested for susceptibility to cleavage by purified granzyume B. The efficiency of cleavage of the substrates by purified granzyme B was also noted (Table 3). Initially, a series of autoantibodies of well-defined specificity were used to immunoblot lysates of HeLa cells that had been incubated *in vitro* with or without granzyme B. Lysates were pre-treated with iodoacetamide (IAA) to prevent interference by endogenous caspase activity.

Interestingly, several autoantigens that have previously been shown to be cleaved by caspases during apoptosis were also efficiently cleaved by granzyme B. These substrates included U1-70kDa, topoisomerase-1, SRP-72, PARP and NOR-90. In each case, unique fragments were generated. Mi-2, PMS2 and Ki-67 were also identified as additional autoantigens that are cleaved into distinct, different sets of fragments both by caspases and by granzyme B in these lysates.

Efficient cleavage and generation of novel fragments was also confirmed using purified granzyme B to cleave *in vitro* translated substrates in the case of U1-70kDa, PARP, topoisomerase-1, PMS2, and Mi-2. The cleavage efficiency (k_{cat}/K_m) of those substrates by granzyme B was determined using defined amounts of purified protease to cleave either endogenous substrates in cell lysates or radiolabeled substrates expressed by *in vitro* transcription/translation, or both. Previous studies have demonstrated that equivalent results are obtained using either form of substrate (Andrade *et al.*, 1998). k_{cat}/K_m values varied between 1.39×10^4 $M^{-1}.s^{-1}$ (PMS2) and 1.6×10^6 $M^{-1}.s^{-1}$ (topoisomerase-1) (see Table 3).

Previous studies have identified several autoantigens that are not susceptible to cleavage by caspases during apoptosis (Casciola-Rosen *et al.*, 1995 & 1996). Many of these autoantigens were efficiently cleaved by granzyme B. These molecules included fibrillarin, PMS1, CENP-B, Ku-70, La and RNA polymerase II large subunit. The efficiency of cleavage of these substrates by granzyme B varied between 5.9×10^3 $M^{-1}.s^{-1}$

(CENP-B) and 8 x 10^4 $M^{-1}.s^{-1}$ (La) (See Table 3). Interestingly, several ribonucleoprotein autoantigens were not susceptible to cleavage by either granzyme B or caspases. These included Ro52kDa and 60kDa, ribosomal protein P, histones and Sm proteins.

Susceptibility to cleavage by granzyme B was a highly specific feature of autoantigens. None of 18 different human non-autoantigens tested was cleaved by granzyme B. The precursors of caspases 3 and 7 are not known to be autoantigens, but are efficiently cleaved by granzyme B. Interestingly, these substrates are cleaved at the same sites by granzyme B and caspase-8, generating identical fragments (see below).

Since some well-defined autoantibodies do not recognize their antigens by immunoblotting, the susceptibility of radiolabeled endogenous substrates to cleavage by granzyme B *in vitro* was assessed. To perform these studies, HeLa cells were radiolabeled with [^{35}S] methionine/cysteine, and proteins were immunoprecipitated using human autoantibodies. Protein A-agarose beads containing washed precipitated proteins were resuspended in buffer supporting the activity of granzyme B, and incubated in the absence or presence of added purified granzyme B. Reaction products were visualized using SDS-PAGE and fluorography. To confirm the validity of this approach, several different autoantigens known to be cleaved by granzyme B, as well as several autoantigens and non-autoantigens that are not cleaved by granzyme B, were tested. Using autoantibodies that both immunoblot and immunprecipitate, the cleavage profiles obtained using granzyme B to cleave molecules in lysates (followed by detection with immunoblotting), and after immunoprecipitation (from [^{35}S]methionine-labeled HeLa lysate) were compared. Identical results were obtained using these 2 methods for cleaved autoantigens (topoisomerase-1, Mi-2, RNA polymerase II large subunit, Ku-70, PARP, and NOR-90), uncleaved autoantigens (Ku-80, Ro 60k), and control substrates (β-tubulin, vinculin). Using this immunoprecipitation approach, it was demonstrated that several additional autoantigens (PMScl, RNA polymerase I large subunit, histidyl tRNA synthetase, isoleucyl tRNA synthetase and alanyl tRNA synthetase) were cleaved by

granzyme B, generating unique fragments (Table 3). Of note, all these additional autoantigens (with the exception of RNA polymerase I) are targeted in autoimmune myositis. However, two other tRNA synthetases (threonyl tRNA synthetase and glycyl tRNA synthetase) that are also autoantigens in autoimmune myositis, were not cleaved using this approach.

Thus, in addition to the three autoantigens described above to be cleaved by both caspase-3 and granzyme B, these results identify an additional 7 autoantigens have been identified that are cleaved by both proteases but at different sites. Furthermore, another 10 autoantigens are cleaved exclusively by granzyme B, and not by caspases. Therefore, 20 autoantigens targeted across the spectrum of human systemic autoimmune diseases are efficiently cleaved by granzyme B, generating unique fragments not observed during other forms of cell death. (Table 3).

To confirm that similar autoantigen fragments are generated in intact cells during granule-induced cell death, K562 cells were exposed to YT cell granule contents in the presence of Ca^{2+}, and the biochemical status of the autoantigens were analyzed by immunoblotting. In those cases where autoantigens are substrates for both caspases and granzyme B, both fragments were generated (U1-70kDa, PARP, Mi-2, topoisomerase-1, Ki-67). Autoantigens known to be cleaved only by granzyme B were indeed cleaved in the K562/YT granule system and the granzyme B-specific fragments of Ku-70, PMS-1 and RNA polymerase II large subunit were generated.

It was shown that granzyme B-specific fragments of DNA-PKcs are generated during cytotoxic lymphocyte granule-induced target cell death. A similar approach was used to determined whether other autoantigens cleaved by granzyme B *in vitro* are also cleaved during killing of intact Fas-negative target cells by lymphokine-activated killer (LAK) cells. Granzyme B-specific fragments of Mi-2, U1-70kDa, topoisomerase-1, PMS-1 and SRP-72 as well as Ku-70, RNA polymerase II and Ki-67, are generated during this form of cell death and were identified by immunoblotting with appropriate antibodies. In the cases of Mi-2, U1-70kDa, SRP-72 and topoisomerase-1, which are all

susceptible to direct cleavage by both caspase-3 and granzyme B, the amounts of granzyme B-specific fragments of these antigens appear to be determined by the relative efficiency of cleavage by the two proteases. Thus, granzyme B-specific fragments of Mi-2 and topoisomerase-1 (which are efficiently cleaved by granzyme B) were clearly observed. In contrast, very low levels of granzyme B-specific fragments of U1-70kDa, SRP-72 or the less efficiently cleaved sites on toposiomerase-1 (72 and 75kDa) were observed in the intact cell killing assay unless caspases were inhibited by adding Ac-DEVD-CHO. The cleavage of PMS1, which is a substrate for granzyme B but not for caspases, was unaffected by Ac-DEVD-CHO.

EXAMPLE 16

Specificity of Granzyme B Clevage of Autoantigens

Granzyme B is a serine protease whose specificity has been defined using a positional scanning combinatorial tetrapeptide library. The protease has a preference for I,V or L in P_4, E, G, S in P_3, and P, S, N, A, Q, H, T, V, E, D, in P_2, with a preference for D in P_1. The sizes of the fragments generated by granzyme B cleavage and the cleavage specificity was used to identify likely cleavage sites. Since granzyme B prefers Asp in the P_1 position, and does not tolerate Ala at that site, site-directed mutagenesis was employed to make a series of Asp-Ala substitutions in several of the granzyme B substrates. The effects of each mutation on the efficiency of cleavage by the protease was assessed.

The granzyme B cleavage sites in PARP and DNA-PKcs have been defined. Using the above approach, the granzyme B cleavage sites in fibrillarin, Mi-2, topoisomerase 1, PMS1, PMS2, and U1-70kDa were also defined (Table 3). Interestingly, 10 of 11 of these cleavage sites contain P (7), A (2), or S (1) in the P_2 position, which are preferred by granzyme B but are poorly tolerated by group III caspases. Furthermore, 4 cleavage sites also contain G or S in P_3. These residues are also not tolerated by group III caspases. Using fragment sizes to predict likely granzyme B cleavage sites in other autoantigens, likely

cleavage sites were also identified in these proteins. In every case, these cleavage sites contained residues in P_2 and/or P_3 which are preferred by granzyme B, but are not tolerated by group III caspases (Table 3).

To confirm that these substrates were not cleaved by group III caspases, the substrates were incubated with 50nM purified caspase-8. Cleavage assays were performed in HeLa cell lysates in which endogenous caspases had first been irreversibly inactivated by 1mM iodoacetamide (4°C for 15 min), prior to addition of 5mM DTT to facilitate exogenous caspase-8 activity. No cleavage of topoisomerase-1, Mi-2, U1-70kDa, PARP, Ku-70, RNA polymerase II large subunit, SRP-72, NuMA or Ki-67 occurred. Caspase-8 was indeed active in these lysates, as evidenced by robust caspase-3 cleavage products of PARP and U1-70kDa seen when the IAA 'poisoning' step was omitted. Radiolabeled PMS1 and PMS2 (generated by IVTT) were also not cleaved by purified caspase-8.

It was demonstrated that 20 of 28 autoantigens tested were susceptible to efficient cleavage by granzyme B, generating unique fragments. Although the cleaved molecules differ markedly in subcellular location, function, and extended primary sequence, they share 2 features: (i) all are autoantigens targeted by a high titer autoantibody response in human autoimmune diseases, including SLE, Sjogren's syndrome, diffuse and limited scleroderma, and autoimmune myositis; and (ii) molecules are unified by containing a granzyme B cleavage site not susceptible to cleavage by caspase-8 (see below). Interestingly, autoantibodies against the precursors of caspases 3 and 7 (which are cleaved by granzyme B and caspase-8 cleave at the same sites generating identical fragments) have not been found in >1000 autoimmune sera screened by immunoblotting.

The status of a molecule as an autoantigen and its unique susceptibility to cleavage by granzyme B but not by caspase-8, are therefore highly related ($P<0.0001$; Chi-square analysis). The positive predictive value of susceptibility to unique cleavage by granzyme B and status as an autoantigen is 100% for these 48 substrates, while the negative-predictive value is 73%, indicating that additional mechanisms play a role in selection of some molecules as autoantigens. It is

noteworthy that most of the uncleaved molecules are nucleoprotein complexes (e.g. components of nucleosomes and snRNPs).

The granzyme B cleavage sites in several molecules are highly conserved, even in drosophila and yeast. This striking conservation of sequence at granzyme B cleavage sites in organisms in which cytotoxic lymphocytes had not yet evolved implies that an important, as yet undefined function is served by these regions. This new, extended family of granzyme B substrates therefore provide a powerful tool with which to explore the evolution and biological functions of the aspartic acid-specific apoptotic proteases, and to probe the mechanisms of cytotoxic lymphocyte granule-mediated cell death.

Human systemic autoimmune diseases represent a highly complex disease spectrum, with numerous variables affecting individual susceptibility, initiation, and tissue targets. By demonstrating that the autoantigens targeted across the spectrum of these diseases are unified by their susceptibility to efficient cleavage by granzyme B, with the generation of unique fragments not generated during any other form of cell death, these studies focus attention on the role of the cytotoxic lymphocyte granule pathway in initiation of autoimmunity. Where substrates are cleaved by both caspases and granzyme B, generation of unique granzyme B fragments is dependent on relative inhibition of the caspases. Without wishing to be bound by a particular theory, it is therefore proposed that during pro-immune intracellular infections occurring in a microenvironment in which caspase activity is under relative inhibition, production of unique granzyme B fragments is favored. In susceptible individuals in whom clearance of apoptotic material is impaired, suprathreshold amounts of these fragments accumulate and are effectively captured and presented by dendritic cells. The resulting immune response is directed against products of CTL granule-induced death, generating an autoamplifying injury characteristic of these self-sustaining diseases.

EXAMPLE 17

Screening for Candidate Agents for Treatment

The assays described herein can be adapted for screening for candidate agents for the prophylactic or therapeutic treatment of autoimmune disease, cancer, or the symptoms of such diseases. In an exemplary format, a candidate agent is contacted with both an uncleaved autoantigen and the contents of a lymphocyte granule, a granule enzyme, or granzyme B. The granule enzyme or granzyme B can be prepared in varying degrees of purity. The autoantigen should be a substrate cleavable by the granule contents or, if a purified or partially purified enzyme is used, a substrate for the particular enzyme. Once contacted, one can monitor the cleavage of the autoantigen into autoantigenic fragments. If desired, one can run a control assay with no candidate agent, or a known inhibitor of the enzyme, in parallel. The production of autoantigenic fragments can be monitored by a variety of means known in the art including antibody capture of the epitopes produced through cleavage, the loss of epitopes that span the cleavage site, separation of cleavage products through chromatography or electrophoresis and other techniques known and used in the art or developed subsequently in the art of detection. A screening assay can be quantitative or qualitative.

A candidate agent can be a chemical compound, organic or inorganic, or a biochemical compound including proteins, peptides, glyco-proteins or peptides, polysaccharides or other macromolecules.

A candidate agent that decreases the rate or the amount of cleavage of the autoantigen to autoantigenic fragments is referred to as an inhibitor of the process. Candidate agents can be studied to determine their suitability for application in the treatment of animals and humans by methods and procedure recognized in the art of pharmaceutical sciences. Those candidates which through testing are shown to have appropriate efficacy and an acceptable safety profile are used in the prophylactic or therapeutic treatment of patients.

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TABLE I. Comparison of k_{cat}/K_m (catalytic constant) values for the cleavage of different substrates by granzyme B and caspase-3.

The data obtained in time-course experiments were densitometrically scanned and used to calculate the % cleavage of each substrate. These values were fitted to the first order rate equation [% substrate cleaved = $100*(1-e^{-((kcat*[E]/Km)*time)})$] to calculate k_{cat}/K_m. Measurements for each protease-substrate combination were performed at 3 different protease concentrations, enabling experimental variations in k_{cat}/K_m to be assessed.

Substrate	**Method of Detection**	k_{cat}/K_m ($M^{-1}s^{-1}$)	
		Cleavage by granzyme B	**Cleavage by caspase-3**
DNA-PK_{cs}	Immunoblotting	$2.5 \pm 0.8 \times 10^6$	$7.5 \pm 0.8 \times 10^6$
NuMA	Fluorography	$5.4 \pm 1.5 \times 10^5$	$5.0 \pm 1.0 \times 10^5$
Caspase-7[1]	Fluorography	$1.8 \pm 0.6 \times 10^5$	--
Caspase-7[1]	Immunoblotting	$1.9 \pm 0.6 \times 10^5$	--
Caspase-3	Fluorography	$3.6 \pm 1.0 \times 10^4$	--
Caspase-3	Immunoblotting	$2.3 \pm 0.4 \times 10^4$	--
PARP	Fluorography	$2.3 \pm 1.8 \times 10^4$	$5.0 \pm 0.2 \times 10^6$

[1]The source of caspase 3 and 7 precursors used in immunoblotting was THP^{-1} cytosol. Blotting antibodies were specific for either caspase 3 or 7, respectively.

Table II: Different fragments are detected after *in vitro* cleavage of autoantigens with granzyme B versus caspase-3.

The data obtained in FIG. 2, using purified DNA-PK_{cs}, [^{35}S]methionine-labeled PARP, endogenous DNA-PK_{cs} and NuMA, and purified proteases, were used for the tabulation below.

Substrate	Fragments induced after cleavage with Granzyme B	Caspase-3	Likely granzyme B cleavage sites
DNA-PK_{cs}[2]	100kDa	150kDa	$VDQD^{3210}$-G^{3211}
DNA-PK_{cs}[3]	250kDa	250kDa	$VGPD^{2698}$-F^{2699}; $DEVD^{2712}$-N^{2713}
NuMA	175kDa	185kDa	$VLGD^{411}$-V^{412}
PARP	62kDa	89kDa	$VGPD^{537}$-S^{538} (Froelich *et al.*, 1996a)

2 DNA-PK_{cs} fragments were detected by immunoblotting with monoclonal antibody 25-4 or patient sera A.G. and G.A. (which all recognize the C-terminus).

3 DNA-PK_{cs} fragments were detected by monoclonal antibody 18-2 (which recognizes the N-terminus).

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TABLE 3: AUTOANTIGENS ARE EFFICIENTLY CLEAVED BY GRANZYME B

	Autoantigen	Cleavage site	k_{cat}/K_m ($M^{-1}.s^{-1}$)	Fragments (kDa)
1	DNA-PKcs	$VGPD^{2698}$-F	$2.5 \pm 0.8 \times 10^6$	160,100
2	Topoisomerase I	$I\ EAD^{15}$-F	$1.6 \pm 0.6 \times 10^6$	97,72
3	NuMA	$VATD^{1705}$-A	$5.4 \pm 1.4 \times 10^5$	175
4	Mi-2	$V\ DPD^{1312}$-Y	$8.5 \pm 1.9 \times 10^4$	75,72,48
5	La	$LEED^{220}$-A	$6.1 \pm 1.7 \times 10^4$	21,28
6	PMS1	$LTPD^{313}$-K $ISA\ D^{496}$-E	$6.9 \pm 0.9 \times 10^4$	48
7	Fibrillarin	$VGPD^{184}$-G	$3.3 \pm 1.9 \times 10^4$	20,17
8	PARP	$VDPD^{536}$-S	$2.3 \pm 1.8 \times 10^4$	72,62,55
9	U1-70kDa	$LGND^{409}$-S	$1.3 \pm 0.4 \times 10^4$	60
10	PMS2	$VEKD^{493}$-S	$1.4 \pm 0.6 \times 10^4$	60,45,35
11	Isoleucyl tRNA synthetase (O.J.)	$VTPD^{982}$-Q	$7.8 \pm 1.8 \times 10^4$	
12	Histidyl tRNA synthetase (Jo-1)	$LGPD^{48}$-E	$2.3 \pm 0.7 \times 10^4$	40
13	Alanyl tRNA synthetase (PL-12)	$VAPD^{632}$-R	1.8×10^4	63,40
14	RNA polymerase I	$ICPD^{448}$-M	$1.3 \pm 0.5 \times 10^4$	140
15	KI-67	$VCTD^{1481}$-K	$8.1 \pm 2.6 \times 10^3$	168,148
16	PmScl	$VEQD^{252}$-M	$7.5 \pm 1.4 \times 10^3$	85,74,60
17	CENP B	$VDSD^{457}$-E	$5.9 \pm 0.2 \times 10^3$	58,40
18	RNA polymerase II	$I\ TPD^{370}$-P	ND	200
19	SRP 72	$VTPD^{573}$-P	ND	62
20	Ku 70	$I\ SSD^{79}$-R	ND	65
21	Tyrosinase	$ICTD^{249}$		
22	E4	$VDVD^{123}$		
23	Golgin 160	$VGPD^{92}$ $SEVD^{311}$ $IEAD^{648}$		
24	Myosin			